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14. ABSTRACT

The overarching goal of this proposal is to identify the regulatory networks controlling the patterned regrowth of damaged limbs and limb tissues in vertebrates, and gain insight into the fundamental genetic circuitry directing this complex process. During this 2011-2012 grant year, we have achieved two major goals in our research program. We have performed transcriptome analysis of salamander and *Polypterus* regenerating limbs and identified extracellular matrix (ECM) genes as the most highly upregulated pathway in response to injury. The ECM has been linked to cellular migration but the potential impact during the initiation of regenerative outgrowth, however, remains largely unknown. In addition, we have submitted RNA samples for deep-sequencing experiments to elucidate the contributions of microRNAs as key genetic determinants of positional memory during zebrafish appendage regeneration. We anticipate these microRNAs experiments to yield candidate factors for functional characterization for the duration of the grant period. In summation, we have made notable progress in our efforts to identify the common regulatory pathways required for appendage regeneration and remain on schedule to complete future milestones for grant year 2012-2013.

15. SUBJECT TERMS

limb regeneration	Positional Memory Code	Axolotl
microRNAs	Zebrafish	Polypterus

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Table of Contents

THE CENTER FOR REGENERATIVE BIOLOGY AND MEDICINE AT MOUNT DESERT ISLAND BIOLOGICAL LABORATORY

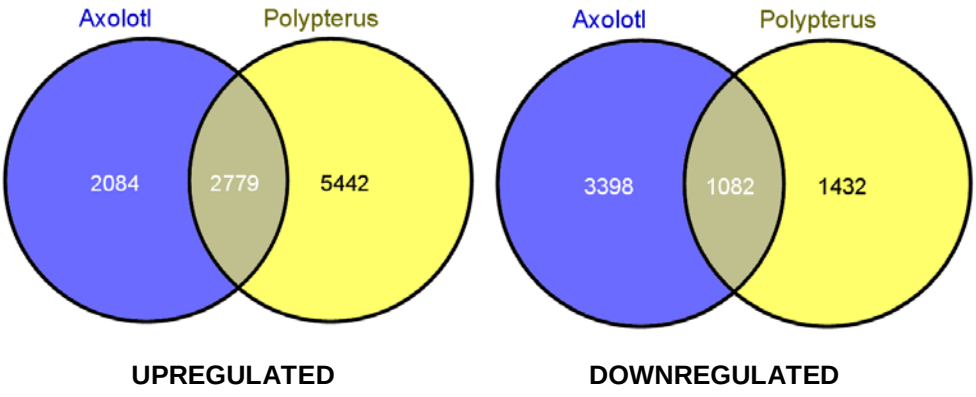
INTRODUCTION:

The inability to regrow functional limbs or limb segments lost to trauma or disease is a significant biomedical problem, with substantial associated monetary and quality-of-life implications for the nearly two million affected U. S. citizens and active service members. Development of *in vivo* therapies that restore regenerative capacity first requires an understanding of the basic gene regulatory networks controlling this biology. Thus, characterizing ancestral regulatory circuitry controlling regeneration is a necessary and direct route to identifying the mechanistic causes of regenerative failure in mammals. This proposal offers unique promise in guiding the targeted development of *in vivo* therapies to restore/augment human limb regeneration. We will leverage our recent discovery that regenerative ability is widespread in basal vertebrates to conduct the first comparative analysis of appendage regeneration that incorporates model systems from all major groups of limbed vertebrates- cartilaginous fishes, ray-finned fishes, and tetrapods. Our unique approach will identify novel functional requirements for genes/gene networks in regulating appendage regeneration by marrying a comparative organismal approach with state-of-the-art systems-level analyses of gene expression using next generation RNA sequencing and functional analysis of candidate regulators in the genetically tractable zebrafish model system through *in vivo* disruption of gene function.

BODY:

Specific Aim 1: Characterize mRNA expression profiles in regenerating salamander (*Ambystoma mexicanum*) limbs and *Polypterus senegalus* fins. This work will be accomplished by **a**) collecting tissue and mRNA from regenerating salamander and *Polypterus* appendages, **b**) characterizing gene expression profiles by DNA sequencing using Solexa/Illumina technology, **c**) assembling and annotating transcriptome sequence, **d**) identifying conserved regulators of limb/fin regeneration by quantitative bioinformatic analysis, and **e**) assessing functional requirements of candidate regulators.

Research Accomplishments: The major focus for Aim 1 during Year 1 was to extract RNA from defined stages of limb regeneration and perform deep sequencing experiments in an effort to identify regulatory programs essential for injury repair. We reasoned that mRNAs that are important for the regenerative response are likely to be differentially regulated in response to injury. Thus, for each species, we compared and contrasted the dataset between uninjured and 7 days post-amputation (dpa) samples, a time of active regenerative growth. To further refine the genes important for regeneration, we applied another selection factor to identify those genes that are similarly controlled in both *Polypterus* and axolotl samples. These comparisons revealed a total of 2779 shared genes that are significantly upregulated during regeneration. Conversely, our analysis showed that 1082 genes are downregulated in response to limb amputation (Figure 1).



UPREGULATED DOWNREGULATED

Figure 1: Venn diagram of UniProt protein sequence IDs among Axolotl and Polypterus contigs that were up-regulated and down regulated greater than 2-fold between 0 and 7 dpa. (Left) The common set of 2,779 upregulated UniProt protein sequence IDs were mapped to 2,617 human Ensembl genes. (Right) The common set of 1,082 downregulated UniProt protein sequence IDs were mapped to 1,019 human Ensembl genes.

From this dataset, we identified the 10 most highly upregulated genes during regeneration in both *Polypterus* and axolotl limb regeneration (Table 1). This collection of genes is involved in remodeling of the extracellular matrix, a process that is critical during cell movement and cellular dedifferentiation. We will select from this group 1-2 genes for further characterizations during limb regeneration. We are currently continuing our analyses with a focus on downregulated genes.

Table 1. Top ten most highly upregulated genes during axolotl and *Polypterus* limb regeneration.

Associated Gene	Description
KRT19	keratin 19 [Source:HGNC Symbol;Acc:6436]
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase) [Source:HGNC Symbol;Acc:7176]
CTSK	cathepsin K [Source:HGNC Symbol;Acc:2536]
MMP13	matrix metallopeptidase 13 (collagenase 3) [Source:HGNC Symbol;Acc:7159]
KRT17	keratin 17 [Source:HGNC Symbol;Acc:6427]
MMP10	matrix metallopeptidase 10 (stromelysin 2) [Source:HGNC Symbol;Acc:7156]
PRDM1	PR domain containing 1, with ZNF domain [Source:HGNC Symbol;Acc:9346]
MMP1	matrix metallopeptidase 1 (interstitial collagenase) [Source:HGNC Symbol;Acc:7155]
NCF2	neutrophil cytosolic factor 2 [Source:HGNC Symbol;Acc:7661]
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase) [Source:HGNC Symbol;Acc:7173]

Specific Aim 2: Characterize microRNA (miRNA) expression profiles in regenerating salamander (*Ambystoma mexicanum*) limbs and *Polypterus senegalus* fins. This work will be accomplished by **a**) collecting tissues and isolating small RNA from regenerating salamander and *Polypterus* appendages, **b**) characterizing miRNA expression profiles generated by DNA sequencing using Solexa/Illumina technology, **c**) annotating miRNAs and characterizing their expression profiles in limb/fin regeneration, **d**) predicting miRNA targets and correlating miRNA expression with predicted targets, and **e**) assessing functional roles of candidate miRNAs.

Research Accomplishments: Gene regulatory networks are controlled at multiple levels. microRNAs are key regulatory factors during gene expression with the unique ability to modulate hundreds of target genes. Toward our goal to understand miRNA contributions during limb/appendage regeneration, we have isolated regenerating tissues from the designated timepoints: 0, 3, 7 and 14 dpa in the same manner as outlined for Specific Aim 1 for both *Polypterus* and axolotl tissues. However, to focus on miRNAs, we enriched our RNA samples for small microRNAs (less than 200 nucleotides) with silica columns (Qiagen). Subsequent quality analyses confirmed that we have obtained high-quality miRNAs from all tissues. We have sent our RNA samples for cDNA library synthesis and deep sequencing for small RNAs.

Specific Aim 3: Determine a genetic positional memory code for appendage regeneration. This work will be accomplished by **a**) isolating tissue and RNA from uninjured and regenerating zebrafish caudal fins, **b**) profiling miRNAs with microarray hybridization technology and **c**) filter and categorize the dataset into increasing and decreasing miRNA expression.

Research Accomplishments: To initiate our studies on positional memory, we have isolated uninjured tissue from specified regions of the zebrafish caudal fin. The amputation planes were designated proximodistal (PD) 1-3 (Figure 2). We collected tissue that was ~2 bony segments directly proximal to the PD amputation plane and isolated total RNA in the second quarter of year 1. To achieve our research goal of identifying candidate miRNAs as regulators of positional memory, we also have collected 4 dpa r egenerating tissues from three distinct amputation planes, PD1-3. All collections were performed in triplicate. We have confirmed with gel electrophoresis and Bioanalyzer studies that we have obtained high quality total RNA, suitable for library preparations and deep sequencing experiments. Our samples have been prepped for cDNA library synthesis followed by deep-sequencing reactions.

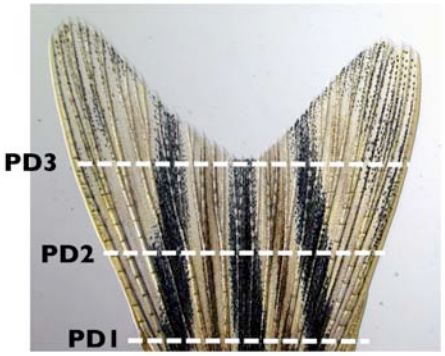


Figure 2. Schematic of positional memory amputation planes. Adult zebrafish caudal fins were independently amputated at proximodistal 1-3 (PD1-3). A second amputation plane, located at ~ 2 bony segments proximal to the original cut sites, were harvested for total RNA isolation.

Specific Aim 4: Define requirements for region-specific regulatory factors in maintaining positional memory. This work will be accomplished by **a**) validating miRNA expression using Northern blot hybridization and/or real-time quantitative PCR, **b**) determining spatial resolution of miRNAs during regenerative states and **c**) performing functional studies on miRNAs using antisense oligonucleotides to determine the effects on regeneration.

Research Accomplishments: We will initiate our studies in Specific Aim 4 in the 2nd quarter of Year 2, as indicated in our table of milestones. Therefore, we do not have results to report at the current time.

Specific Aim 5: Grow Mount Desert Island Biological Laboratory as a collaborative center for the Regenerative Biology and Medicine research community. This work will be accomplished by establishing a MDIBL Visiting Scholars Program in Regenerative Biology.

Research Accomplishments: Our advertisement for Fellowships in Regenerative Biology and Medicine was sent to domestic and international research labs studying regeneration and to department heads at academic medical centers. From the collection of highly competitive applications, the selection committee reviewed and awarded three Regeneration Fellowships to Dr. Malcolm Maden from the University of Florida, Dr. Kenneth Poss from Duke Medical Center and Dr. Jorge Contreras from University of New Jersey School of Medicine and Dentistry. These investigators are scheduled to deliver a scientific seminar during their time at MDIBL.

KEY RESEARCH ACCOMPLISHMENTS:

- Successfully performed transcriptome analysis during *Polypterus* and axolotl limb regeneration.
- Identified regulators of extracellular matrix as the most highly upregulated genetic program in response to injury.
- Successfully isolated small RNA species for deep-sequencing experiments to complement mRNA transcriptome dataset in amphibian studies.
- Completed the isolation, quality validation and have prepped all uninjured and regenerating zebrafish samples along the proximodistal axis for small RNA analyses.
- Successfully recruited and awarded regeneration fellowships to prominent regeneration scientists.

REPORTABLE OUTCOMES:

Poster presentation and abstract at the 39th Maine Biological and Medical Sciences Symposium:

MicroRNAs Let-7i and miR-101a are regulated during zebrafish caudal fin regeneration

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Danio rerio (zebrafish) possess an enhanced capacity to regenerate many tissues relative to mammals. Understanding the cellular and genetic influences for appendage regeneration in zebrafish could provide clues for improving mammal regeneration capacity. MicroRNAs (miRNAs) constitute a class of gene expression regulators with important roles during limb morphogenesis and patterning. In this study, we examined the extent that miRNAs let-7i and miR-101a contribute to appendage regeneration. Caudal fins of adult wild-type zebrafish were amputated 50% from the basal body along the proximodistal axis. Noticeable blastemal formation was detected 2 days post-amputation (dpa). At 4 dpa, images of the regenerating caudal fin showed

extensive blastemal formation and regeneration. Regenerated tissues were isolated for RNA extraction, cDNA synthesis, and subjected to real-time QPCR studies. MiR-101a exhibited increased expression in regenerating tissues at 2 dpa with a fold change of 16, when compared to uninjured tissues. Conversely, let-7i expression decreased at 2, 4, and 14 dpa (fold change 4.7, 3.2 and 3.1, respectively). These data suggest that miRNAs are rapidly modulated in response to injury, implicating important roles during blastemal formation and tissue outgrowth. In future studies, we will manipulate levels of let-7i and miR-101a and quantify changes in regenerative capacity to better understand miRNA impact on appendage regeneration.

This project was supported by grants from the National Center for Research Resources (5P20RR016463) and the National Institute of General Medical Sciences (8 P20 GM103423) from the National Institutes of Health (V.P.Y) and from TARTC and USAMRAA (W81XWH-11-1-0425 (V.P.Y).

Funding applied for based on work supported by this award:

NIH R01. *Genetic Analysis of Cellular Dedifferentiation during Appendage Regeneration.* This grant application will address the roles of miRNAs as key triggers for transforming differentiated tissue into highly proliferative, regenerative cells in response to amputation. 30% effort, April 2013-Mar 2018, Role: PI. Annual direct cost: $250,000, annual indirect cost: $200,000. *Submitted by V.P.Y.*

CONCLUSIONS:

In order to develop potential therapies to restore and/or augment human limb regeneration, we must first understand the molecular regulation of appendage regeneration in vertebrates that have retained enhanced regenerative capacity during evolution. Two critical limitations have impeded progress in this area: 1) lack of diverse experimental animals has precluded the powerful comparative approaches that have vertically advanced other fields of regenerative medicine such as stem cell biology; 2) unbiased functional genomic approaches have not been fully exploited. The experimental design we advance in this proposal integrates two innovative approaches to address these issues, and distinguishes this work as an unique and complementary extension of current projects in TATRC's portfolio. We have and will continue to use a novel comparative approach employing phylogenetically diverse experimental organisms, and adopt unbiased systems-level approaches to identify and dissect the gene networks initiating and maintaining regenerative responses in vertebrate limbs.

In Year 1, we have achieved all milestones outlined in the original research proposal. In brief, a major finding that emerged from our transcriptome analysis of *Polypterus* and axolotl limb regeneration studies is the identification of extracellular matrix (ECM) factors as the most highly upregulated factors. It is our developing hypothesis that the ECM is important in coordinating not only cellular movement but also is vital for cellular dedifferentiation during the initiation of the regenerative response. Therefore, we will conduct functional studies to examine the potential contributions of the ECM for the duration of this grant. It is our expectation that our recent progress and the collective aims of this proposal will provide novel fundamental insight into the molecular control of appendage regeneration, vertically advancing our understanding of the mechanistic causes of regenerative failure in mammals, and ultimately guiding the targeted development of *in vivo* therapies to restore/augment human limb regeneration.

REFERENCES: N/A

APPENDICES: N/A

SUPPORTING DATA: N/A